Isolation and Characterization of Vancomycin-Tolerant *Streptococcus pneumoniae* from the Cerebrospinal Fluid of a Patient Who Developed Recrudescent Meningitis

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The emergence of tolerance to vancomycin has recently been reported in *Streptococcus pneumoniae*, the most common cause of bacterial meningitis. A vancomycin- and cephalosporin-tolerant strain of *S. pneumoniae*, the Tupelo strain, was isolated from the cerebrospinal fluid of a patient who then developed recrudescence of meningitis despite treatment with vancomycin and a third-generation cephalosporin. The Tupelo strain evidenced no lysis in the exponential or stationary phase of growth when exposed to vancomycin and only minimal loss of viability. Further characterization revealed normal autolysin expression, localization, and triggering by detergents, indicating that the defect leading to tolerance in the Tupelo strain is in the control pathway for triggering of autolysis. Because tolerance is a precursor phenotype to resistance and may lead to clinical failure of antibiotic therapy, these observations may have important implications for vancomycin use in infections caused by *S. pneumoniae*.

*Streptococcus pneumoniae* is the most common cause of bacterial meningitis and is associated with the highest case fatality rate, reported as 21% in 1995 [1]. Over the last decade, the percentage of pneumococcal strains isolated from children with invasive infections that are nonsusceptible to penicillin and cephalosporins has increased dramatically [2], leading to recommendations that vancomycin should be included in the initial empiric antibiotic coverage for children with suspected bacterial meningitis [3]. Increased use of vancomycin for invasive *S. pneumoniae* infections may facilitate development of resistance or tolerance to this important drug.

Recently, strains of *S. pneumoniae* tolerant to vancomycin have been characterized [4]. Tolerance is the ability of bacteria to survive in the presence of an antibiotic, neither growing nor undergoing lysis. Because vancomycin- [5] or beta-lactam– [6] tolerant *S. pneumoniae* are difficult to eradicate from the meninges in animal models, this finding may have important implications for the treatment of pneumococcal meningitis. In addition, tolerance may be a precursor background for the development of resistance [4, 7]. We describe here microbiologic properties of a vancomycin-tolerant strain of *S. pneumoniae* isolated from the cerebrospinal fluid (CSF) of a child with meningitis who, despite treatment with vancomycin and third-generation cephalosporins, developed recrudescent meningitis after cessation of therapy.

**Case report.** A previously healthy 10-month-old girl presented to her primary physician in November 1998 with meningitis and bacteremia due to *Streptococcus pneumoniae* (resistant to penicillin and sensitive to ceftriaxone and vancomycin by Kirby-Bauer disk diffusion assay). The CSF had a white blood cell (WBC) count of 1180 cells/mL (76% polymorphonuclear cells and 24% lymphocytes) and a Gram's stain showing gram-positive cocci in pairs and chains. Glucose and protein were not measured on the initial specimen. The patient received 8 days of intravenous cefotaxime (150 mg kg\(^{-1}\) day\(^{-1}\)) and vancomycin (40 mg kg\(^{-1}\) day\(^{-1}\) for 1 day and 68 mg kg\(^{-1}\) day\(^{-1}\) for 7 days, with a peak serum concentration of 31.2 μg/mL) at a local hospital in Tupelo, MS. Dexamethasone was not administered. On hospital day 9 the inpatient antibiotic regimen was changed to daily intramuscular ceftriaxone (100 mg kg\(^{-1}\) day\(^{-1}\)) for the final 2 days of a 10-day course of parenteral antibiotics.

The patient’s course was complicated by a generalized tonic-clonic seizure on hospital day 3, which resolved with anticonvulsant therapy. Repeat lumbar puncture on hospital day 3...
provided CSF with a WBC of 9 cells/mL (100% lymphocytes), a protein level of 30 mg/dL, a glucose level of 45 mg/dL, and a Gram’s stain without bacteria. No organisms grew from this specimen. The patient was discharged on the 11th day after admission and was followed up in an outpatient clinic, during which time the patient had intermittent fevers (temperature, 38.3°C). Eight days after discharge, the patient had recrudescence of meningeal symptoms (fever, irritability, lethargy) and was transferred to Le Bonheur Children’s Medical Center in Memphis. Lumbar puncture prior to transfer provided CSF with a WBC of 213 cells/mL (64% polymorphonuclear cells and 36% lymphocytes), a protein level of 32 mg/dL, and a glucose level of 43 mg/dL. No Gram’s stain was performed due to paucity of the sample. No organisms grew from this or a subsequent CSF sample. The patient was treated with 10 days of cefotaxime (300 mg kg⁻¹day⁻¹), vancomycin (60 mg kg⁻¹day⁻¹) and rifampin (20 mg kg⁻¹day⁻¹) with resolution of meningitis. Findings from computed tomography of the head, magnetic resonance imaging of the head and spine, hearing testing, serum immunoglobulins, and a serum ELISA for human immunodeficiency virus were normal. She has no apparent residual damage 8 months after completion of therapy.

Methods

The clinical strain used in this study, *S. pneumoniae* strain Tupelo, serotype 14, was grown from the original CSF specimen taken from the patient at onset of her meningitis. No additional CSF was available from subsequent lumbar punctures done at the outside hospital, including the lumbar puncture done when the patient had recrudescence of her meningitis. The patient’s strain; another clinical isolate, Norway T4, serotype 4; and the laboratory strains R6 (a derivative of *S. pneumoniae* D39, serotype 2) and Lyt-4-4 (an autolysin-defective isogenic mutant of R6), obtained from the Rockefeller University collection [8], were grown on tryptic soy agar (TSA; Difco, Detroit) supplemented with sheep blood to a final concentration of 3% (v/v). For growth in liquid culture, the bacteria were grown in a semi-synthetic casein hydrolysate medium supplemented with yeast extract (C+Y) [9].

Testing for susceptibility to antimicrobial agents and determining autolysis rates. Susceptibility of the clinical isolate to penicillin, ceftriaxone, erythromycin, azithromycin, trimethoprim-sulfamethoxazole, and vancomycin was determined per the manufacturer’s instructions by use of the E test (AB BIODISK, Solna, Sweden) on bacteria grown on Mueller Hinton agar with 5% defibrinated sheep blood added.

Autolysis rates and viability of the clinical strain were determined by use of 10-mL cultures of *S. pneumoniae* exposed to 10–20 times the MIC of antimicrobial agents, either vancomycin (10 μg/mL) or penicillin (20 μg/mL), when the optical density at 620 nm (OD₆₂₀nm) reached 0.25 to 0.3 (corresponding to 5 × 10⁷ cfu/mL). After various times of exposure, 100-μL portions were removed, serially diluted in C+Y medium, and plated for viability counts on TSA supplemented with 3% sheep blood (v/v). Turbidity was measured hourly for 6 h.

Crude preparation of autolysin and assay for autolytic activity.

Purification of pneumococcal autolysin (LytA) from the Tupelo strain was performed on the basis of an established protocol [10]. In brief, 500 mL of late exponential-phase bacteria was harvested by centrifugation at 5000 g for 10 min. The bacterial pellet was resuspended in 10 mL of ice-cold 20 mM KH₂PO₄ and shock-frozen in an ethanol-ice mixture. After slow thawing, the samples were sonicated to break the cell wall and then centrifuged at 30,000 g for 30 min. The amount of protein in the supernatant (containing the autolysin) was determined by the Bradford test. The activity of autolysin obtained by the method described was determined in a biological assay as follows: The lysis-defective strain Lyt-4-4 was grown to an OD₆₂₀nm of 0.25 and exposed to 10 times the MIC of penicillin (0.1 μg/mL) and crude autolysin preparations. The turbidity readings of the cultures were measured hourly for 6 h, incubating the strains at 37°C and 5% CO₂.

Immunoblotting. LytA was analyzed by subjecting lysates to 10% SDS PAGE and by use of Western blotting with Immobilon-P membranes (Millipore, Bedford, MA), following which 30 μg of protein was loaded on the SDS gel. This quantity was within the linear range. The membranes were incubated with polyclonal rabbit anti-recombinant autolysin antibody (1 : 1000) and developed by use of goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) and an ECL chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom).

Transmission and scanning electron microscopy. Localization of LytA within the bacteria was assessed by transmission and scanning electron microscopy. Bacteria were grown overnight in C + Y medium to an OD₆₂₀nm of 0.5 and fixed in 0.1 M cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde. After this procedure, bacteria were postfixed in 1% osmium tetroxide, dehydrated in gradients of ethanol, stained with 2% uranylacetate, and embedded in Spurr’s resin. Sections were stained with 2% uranylacetate and Reynold’s lead citrate and were examined with a Jeol 1200 EX II electron microscope (Jeol Ltd., Tokyo).

Results

Resistance to antimicrobial agents in strain Tupelo. *S. pneumoniae* strain Tupelo was recovered from the patient’s CSF sample obtained on 30 October 1998. The isolate was resistant to penicillin with a MIC of 2.0 μg/mL, as determined by the E test method. It was nonsusceptible to ceftriaxone with a MIC of 0.75 μg/mL and was resistant to erythromycin, azithromycin, and trimethoprim-sulfamethoxazole. Initial susceptibility to vancomycin was determined by disk diffusion. MIC, as determined by the E test method in our laboratory, was 0.5 μg/mL.

Rate of vancomycin-induced killing in the Tupelo strain. Because the organism had been cultured from a patient who subsequently developed recrudescence of her meningitis, antibiotic tolerance to vancomycin and the cephalosporins was suspected. To assess whether tolerance was present, the effect of vancomycin on turbidity and viability was examined. After 1 h, a nontolerant, penicillin-susceptible clinical isolate, Norway T4, and laboratory strain, R6, had undergone vancomycin-induced lysis, whereas over a period of 6 h, the Tupelo strain demonstrated no significant indication of lysis (figure 1A). In
addition, almost no lysis was observed in the stationary phase of growth. A substantial loss of viability was demonstrated for the nontolerant clinical isolates after exposure to vancomycin (4 log), whereas a loss of only 2.4 log was observed for the Tupelo strain (figure 1B). Analysis of growth rates showed that the Tupelo strain and the control strains had similar growth rates, demonstrating a generation time of about 40 min during the midlogarithmic phase, which suggested that the tolerant phenotype was not due to differences in growth behavior. Light microscopy showed that the clinical isolate, Norway T4, and the Tupelo strain formed chains of ~10 pneumococci. Although this phenomenon could contribute to a misleading determination of viable numbers, addition of vancomycin to the Tupelo strain led to a significant reduction of chain length, resulting in a predominantly diplococcal appearance (data not shown). The Tupelo strain was tolerant to both penicillin and cefotaxime, with lysis-and-kill curves similar to those seen in figure 1 (data not shown).

**Analysis of the pneumococcal amidase, LytA.** After demonstrating that the isolate was tolerant, we attempted to elucidate the defect or change in function leading to the tolerant phenotype. The reduced lytic and killing effects of vancomycin in the Tupelo strain could be due to changes in the expression, location, or activity of the autolysin, LytA. Western blot analysis of an autolysin preparation of the Tupelo strain showed a normal translational product of LytA (figure 2A). The function of the autolysin was assessed by the ability of the Tupelo autolysin versus the exogenous recombinant autolysin to reconstitute vancomycin-induced lysis of the autolysin-deficient strain Lyt-4-4. The autolysin preparation of the Tupelo strain reconstituted lysis of the Lyt-4-4 strain to the same degree as the wild-type enzyme (figure 2A). These results suggest that lack of autolysis of the Tupelo strain on addition of vancomycin is due to an alteration in the control of the autolytic pathway rather than in the autolysin itself.

*S. pneumoniae* undergoes rapid lysis on exposure to deoxycholate by triggering the LytA activity. As expected, the Tupelo strain was as sensitive to lysis by deoxycholate as other clinical isolates are sensitive to penicillin and vancomycin (data not shown). Immune electron microscopy of the Tupelo strain confirmed the association of the autolysin with the cell wall, excluding the remote possibility of an autolytic dysfunction due to a change in target localization (figure 2B).

**Discussion**

The emergence of vancomycin-tolerant clinical strains of *S. pneumoniae* has recently been reported [4, 5]. Three clinical samples have been described: 2 strains isolated from the nasopharynx and 1 strain from an invasive bloodstream infection, all serotype 9V [5]. We describe here the first vancomycin-tolerant strain of *S. pneumoniae* isolated from the CSF of a patient with meningitis. The isolate was tolerant to the effects of vancomycin, penicillin, and the cephalosporins, failing to undergo lysis when exposed to these antibiotics in vitro. The patient developed recrudescent meningitis 8 days after receiving a 10-day course of antibiotics for meningitis, including third-generation cephalosporins and vancomycin.

The bactericidal activity of antibiotics that interrupt cell-wall synthesis relies heavily on the autolytic enzyme, which can be
triggered to digest the cell-wall exoskeleton by antibiotics [11, 12]. The ability to survive exposure to an antibiotic through loss of autolysin activity or triggering is termed “tolerance.” Given normal autolysin expression, localization, and triggering by detergents, the defect leading to tolerance in the Tupelo strain appears to be in the control pathway for triggering of autolysis. This defect prevents lysis of the bacterium in the presence of vancomycin, penicillin, or the cephalosporins. The strain was tolerant to penicillin and the cephalosporins, even at doses well above its elevated MIC.

Although tolerance to vancomycin has only recently been described in *S. pneumoniae*, tolerance to penicillin has been observed for >10 years [6]. There is a dissociation between tolerance and resistance to penicillin; that is, some strains may be susceptible to penicillin yet tolerant, or vice versa [6]. Thus, the knowledge that an isolate is susceptible to an antibiotic does not provide information on the isolate’s tolerance and may lead to suboptimal therapy. Currently, clinical laboratories cannot detect antibiotic-tolerant strains without performing cumbersome lysis-and–kill curves. Although it has been suggested that determining the strain’s MBC : MIC ratio might be useful to estimate whether or not a strain is tolerant, this method is also cumbersome and is less accurate and reproducible than lysis-and–kill curves. Such tolerant strains may be more difficult to eradicate, requiring prolonged courses of antibiotics [6], particularly in the meninges, where host defenses are poor [5].

The patient reported here had meningitis due to a vancomycin–tolerant strain, with few complications and no obvious residual damage. However, the patient then developed recrudescence of her meningitis, despite 8 days of vancomycin therapy and 10 days of cephalosporin treatment. It is possible that the vancomycin- and cephalosporin-tolerant phenotype of the organism was responsible for the recrudescent symptoms by allowing the bacterium to survive the antibiotic therapy and then rebound. One might speculate that the lack of severe symptoms in this patient was related to the lysis defect, resulting in a lack of a marked inflammatory response on exposure to antibiotics: 3 days after each presentation with meningitis, lumbar punctures yielded CSF with remarkably few cells and normal glucose and protein levels. However, the organism was not isolated from the CSF specimen obtained at the outside institution on recrudescence of her meningitis, and that CSF sample was not available for our study.

It has been suggested that tolerance may be a precursor background to the development of drug resistance because it creates survivors of antibiotic therapy [4, 7]. If this is true, the increased use of vancomycin in clinical situations such as meningitis because of penicillin- or cephalosporin-resistant strains may favor the development of resistant isolates. Vancomycin remains the antibiotic of last resort for the treatment of meningitis caused by pneumococci resistant to beta-lactam antibiotics, and it is now recommended along with cefotaxime or ceftriaxone for the empiric treatment of suspected pneumococcal meningitis (pending susceptibility results). Our finding of vancomycin tolerance in a pneumococcus isolated from the CSF of a child with recrudescent meningitis is cause for concern. We suggest that increased surveillance for such tolerant isolates and more
study of the mechanism(s) responsible for this phenotype are warranted.

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References